

FEB 26 2009

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Shughart<sub>PC</sub>****Fax Information Sheet****DATE:** February 26, 2009

<b>TO: EXAMINER RICHARD A SCHNIZER</b>	<b>FAX NO.: 571-273-8300</b>
United States Patent and Trademark Office	Art Unit 1635; Conf. No. 3738

**FROM:** Paul A. Jenny, Reg. No. 59,014 on behalf of Teddy C. Scott Reg No. 53,573**RE:** Proposed Claim Amendments for U.S. Patent Appl. No. 10/709,739**CLIENT/MATTER NO.:** 050992.0302.00USCP**Number of pages including cover sheet:** 11

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If you do not receive all pages of this communication, please call Paul A. Jenny at (312) 819-1900.

**Message**

Examiner Schnizer,

In preparation for today's telephone interview at 2PM (Eastern) regarding U.S. 10/709,739, please find attached the following:

(1) proposed claim amendments

(2) Murphy et al., which shows that miR 112-1 suppresses expression of the gene encoding immediate-early (IE) protein 1, which corroborates the disclosure at Lines 6,493,606-6,493,610 of Table 7 disclosing the regulation of NC\_001347 (i.e., IRS1 gene) by GAM501831.

Best regards,  
Teddy Scott Jr., Ph.D. 53,573

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FEB 26 2009

Application No. 10/709,739

Docket No. 050992.302.00USNP

**ALTERNATIVE 1: PROPOSED AMENDMENTS TO THE CLAIMS FOR U.S. PATENT APPL.  
NO. 10/709,739**

1. - 25 (Canceled)

26. (Previously Presented) An isolated nucleic acid, wherein the sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 4204050;
- (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
- (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).

27. - 30 (Canceled)

31. (Currently Amended) A vector comprising a heterologous sequence, wherein the heterologous sequence consists of the sequence ~~viral insert, wherein the viral insert consists of the nucleic acid of claim 26, and wherein the vector does not comprise any viral insert other than the nucleic acid of claim 26.~~

32. (Canceled)

33. (Currently Amended) A probe comprising a heterologous sequence, wherein the heterologous sequence consists of the sequence ~~viral insert, wherein the viral insert consists of the nucleic acid of claim 26, and wherein the probe does not comprise any viral insert other than the nucleic acid of claim 26.~~

34. (Canceled)

35. (Previously Presented) An isolated nucleic acid, wherein the sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 117937;
- (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
- (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).

36. (Currently Amended) A vector comprising a heterologous sequence, wherein the heterologous sequence consists of the sequence ~~viral insert, wherein the viral insert consists of the nucleic acid of claim 35, and wherein the vector does not comprise any viral insert other than the nucleic acid of claim 35.~~

Application No. 10/709,739

Docket No. 050992.302.00USNP

37. (Currently Amended) A probe comprising a heterologous sequence, wherein the heterologous sequence consists of the sequence ~~viral insert, wherein the viral insert consists of the nucleic acid of claim 35, and wherein the probe does not comprise any viral insert other than the nucleic acid of claim 35.~~

38. - 40. (Canceled)

Docket No. 050992.0302.00USCP

**ALTERNATIVE 2: PROPOSED AMENDMENTS TO THE CLAIMS FOR U.S. PAT. APPL. NO.  
10/709,739**

1. - 25 (Canceled)

26. (Previously Presented) An isolated nucleic acid, wherein the sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 4204050;
- (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
- (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).

27. - 30 (Canceled)

31. (Currently Amended) A vector comprising a heterologous sequence of a nucleic acid, wherein the heterologous sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 4204050
- (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
- (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).

~~viral insert, wherein the viral insert consists of a nucleic acid consisting of, and wherein the vector does not comprise any viral insert other than the nucleic acid of claim 26.~~

32. (Canceled)

33. (Currently Amended) A probe comprising a heterologous sequence of a nucleic acid, wherein the heterologous sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 4204050
- (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
- (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).

~~probe insert, wherein the probe insert consists of a nucleic acid consisting of, and wherein the probe does not comprise any viral insert other than the nucleic acid of claim~~

~~26.~~

34. (Canceled)

Application No. 10/709,739

Docket No. 050992.302.00USCP

35. (Previously Presented) An isolated nucleic acid, wherein the sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 117937;
- (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
- (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).

36. (Currently Amended) A vector comprising a heterologous sequence of a nucleic acid, wherein the heterologous sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 117937
  - (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
  - (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).
- ~~viral insert, wherein the viral insert consists of a nucleic acid consisting of, and wherein the vector does not comprise any viral insert other than the nucleic acid of claim 35.~~

37. (Currently Amended) A probe comprising a heterologous sequence of a nucleic acid, wherein the heterologous sequence of the nucleic acid consists of:

- (a) SEQ ID NO: 117937
  - (b) a DNA encoding the nucleic acid of (a), wherein the DNA is identical in length to (a); or
  - (c) the complement of (a) or (b), wherein the complement is identical in length to the nucleic acid of (a) or (b).
- ~~probe insert, wherein the probe insert consists of a nucleic acid consisting of, and wherein the vector does not comprise any viral insert other than the nucleic acid of claim 35.~~

38. - 40. (Canceled)

# Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: Implications for latency

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Contributed by Arnold J. Levine, December 19, 2007 (sent for review November 20, 2007)

A quantitative algorithm was developed and applied to predict target genes of microRNAs encoded by herpesviruses. Although there is almost no conservation among microRNAs of different herpesvirus subfamilies, a common pattern of regulation emerged. The algorithm predicts that herpes simplex virus 1, human cytomegalovirus, Epstein-Barr virus, and Kaposi's sarcoma-associated herpesvirus all employ microRNAs to suppress expression of their own genes, including their immediate-early genes. In the case of human cytomegalovirus, a virus-coded microRNA, miR-112-1, was predicted to target the viral immediate-early protein 1 mRNA. To test this prediction, mutant viruses were generated that were unable to express the microRNA, or encoded an immediate-early 1 mRNA lacking its target site. Analysis of RNA and protein within infected cells demonstrated that miR-UL112-1 inhibits expression of the major immediate-early protein. We propose that herpesviruses use microRNA-mediated suppression of immediate-early genes as part of their strategy to enter and maintain latency.

miRNAs | reactivation | immune evasion

**M**icroRNAs (miRNAs) are 20–23-nucleotide RNA molecules that bind to mRNA targets, generally within their 3' untranslated region (3' UTR), and interfere with their translation (1, 2). Viruses have co-evolved with cellular miRNAs and many encode their own miRNAs (3). Every herpesvirus genome that has been examined has been found to encode multiple miRNAs, including Epstein-Barr virus (EBV) (4–6), human cytomegalovirus (HCMV) (4, 7, 8), herpes simplex virus 1 (HSV-1) (9, 10), and Kaposi's sarcoma-associated herpesvirus (KSHV) (4, 6, 11, 12). These miRNAs can potentially function during lytic replication and latency. Lytic replication proceeds in a coordinated three-phase cascade: immediate-early (IE), early and late. IE products prepare the cell for infection and propagate the cascade. Early gene products support replication of viral DNA, and DNA replication is, in turn, a prerequisite for full activation of the late genes that encode the structural proteins of the virus. During latency, the virus is quiescent. A limited subset of the viral genome is expressed, but, importantly, the virus has the potential to reactivate and reenter the lytic cycle. Although the molecular mechanisms of reactivation are not understood, it is widely assumed that the lytic cascade is reinitiated with the expression of IE genes. Ectopic expression of a single IE protein has been shown to reactivate HSV-1 (13, 14), EBV (15), or KSHV (16, 17) in cell culture models of latency.

Whereas none or only a few protein-coding genes are expressed, multiple miRNAs are transcribed during latency. The HSV-1 miR-LAT lies within one of the latency associated transcripts (LATs), the only viral RNAs known to be expressed in latency. EBV and KSHV miRNAs also are expressed during latent infection. Because they are nonimmunogenic, miRNAs should be optimal agents for suppression of anti-viral responses and to modify behaviors of latently infected cells, and recent reports suggest this is the case. The HSV-1 miR-LAT has been reported to inhibit apoptosis in neurons by inhibiting translation

of TGF- $\beta$  and SMAD3 (10); the HCMV miR-UL112-1 represses expression of MICB, a ligand of the natural killer cell activating receptor NKG2D (18); and KSHV miRNAs down-regulate thrombospondin 1 expression, a regulator of cell adhesion, migration and angiogenesis (19).

Virus-coded miRNAs also could act to antagonize the expression of IE genes. This could contribute to down-regulation of IE expression during later phases of the lytic replication cycle when miRNAs have accumulated to relatively high levels, and it could help to establish latency and subsequently antagonize reactivation of latent virus, given the critical role of IE gene products in the initiation of the lytic cascade. Motivated by this reasoning, we developed an miRNA-target-predicting algorithm and applied it to all 3' UTRs of four herpesviruses. The high probability targets predicted by this algorithm included IE transactivators for all four viruses: ICP0 of HSV-1; BZLF1 and BRLF1 of EBV; IE1 of HCMV; Rta and Zta of KSHV. We experimentally confirmed the ability of an HCMV-coded miRNA to reduce IE1 expression. Because these genes are necessary for efficient initiation of the lytic program, we propose that herpesviruses use a common miRNA-based strategy for maintaining latency.

## Results

For further details, please see supporting information (SI) *Materials and Methods* and SI Tables 3–8.

### Predicting Targets of HCMV-Coded miRNAs Within the HCMV Genome.

To test our hypothesis that herpesvirus miRNAs might inhibit expression of viral genes needed for efficient lytic replication and thereby favor latency, we asked whether viral miRNAs had potential to target viral 3' UTRs. Instead of listing all conserved potential miRNA binding sites or computing scores based on various empirical rules, our algorithm uses a combination of analytical expressions and Monte Carlo simulations to determine exact probabilities that predicted miRNA targets would occur by chance. We use the standard assumption that the 3' UTR sequence has coevolved with the sequence of the miRNA (20, 21) and the experimental observation that miRNA binding requires a perfect complementarity of a "seed" sequence near the 5' end of the miRNA to a sequence in the 3' UTR (2). This seed is usually a heptamer at positions 2–8 from the 5' end of the miRNA. As a result of coevolution, the number of actual seed oligomers present in the 3' UTR of a

Author contributions: E.M. and J.V. contributed equally to this work; E.M., J.V., T.S., and A.J.L. designed research; E.M. and J.V. performed research; H.R. contributed new reagents/analytic tools; E.M., J.V., T.S., and A.J.L. analyzed data; and E.M., J.V., T.S., and A.J.L. wrote the paper.

The authors declare no conflict of interest.

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Table 1. Top 10 predicted miRNA-target pairs in HCMV when sorted by  $PV_{SH}$  score

3'UTR		First-order local MM				
HCMV ORF	Length*	hcmv-miR	Act.†	Exp.‡	Log <sub>10</sub> $PV_{SH}$	$PV_{MH}$
UL103	21	US5-2	1	0.000	-4.11	0.036
UL112-113	67	UL54-1	1	0.001	-2.96	0.155
RL10	57	US5-1	1	0.003	-2.52	0.273
UL31	62	UL112-1	1	0.003	-2.46	0.229
UL80	34	UL70-5p	1	0.004	-2.42	0.187
UL34	14	UL112-1	1	0.004	-2.41	0.155
UL3	57	UL70-5p	1	0.005	-2.33	0.155
UL69	253	US33-1	1	0.005	-2.29	0.144
UL57	426	US25-2-5p	2	0.108	-2.27	0.127
<b>UL123(IE1)</b>	<b>92</b>	<b>UL112-1</b>	<b>1</b>	<b>0.006</b>	<b>-2.21</b>	<b>0.125</b>

The table shows the top 10 of 4,896 possible miRNA-3' UTR pairs for the HCMV genome. The statistical significance of the top targets is measured by the multiple hypothesis  $P$  value  $PV_{MH}$ . The random background used is the first-order local MM. IE1 (UL123) is shown in bold.

\*Length denotes the total number of all conserved heptamers in the 3' UTR.

†Act. denotes the actual count (in the 3' UTR) of conserved heptamers complementary to the miRNA seed.

‡Exp. denotes the count expected in the random sequence.

targeted gene will be higher than the number that would appear by chance in a random sequence with similar composition. The algorithm predicts functional miRNA targets in two steps.

First, for each miRNA-3' UTR pair, we compute an approximate probability  $PV_{SH}$  ( $P$  value for single hypothesis testing) that it would appear by chance in the random sequence; the smaller  $PV_{SH}$  is, the more likely the given pair is to be biologically functional. (Probability  $PV_{SH}$  is very nearly exact. The only approximation is that we assume independence between consecutive oligomers.) This procedure alone allows testing whether a given miRNA is likely to target a given 3' UTR and is analogous to that used by Robins and Press (21).

Second, if we are interested in finding functional targets of multiple miRNAs among multiple 3' UTRs, we need to take into account multiple hypothesis testing. We do this by performing a Monte Carlo simulation in which we compute the probability  $PV_{MH}$  ( $P$  value for multiple hypothesis testing) that the top, say 10, miRNA-target pairs in a randomly generated genome with similar properties would have their  $PV_{SH}$  lower than the corresponding top 10 miRNA-target pairs in the real genome. We used this approach instead of the now standard False Discovery Rate analysis (FDR) of Benjamini and Hochberg (22) because of the discrete nature of our data. In our case, most  $PV_{SH}$  values are 1, and so FDR analysis is not applicable because it requires a fairly uniform distribution of  $PV_{SH}$  except a small overrepresentation at values close to 0.

Details and various extensions of the algorithm, such as the generation of the random background sequence based on the Markov model (MM), the calculation of the single and multiple hypothesis  $P$  values, the use of conservation (21) among strains of the virus, and the choice of the seed sequence, are described in *Materials and Methods*.

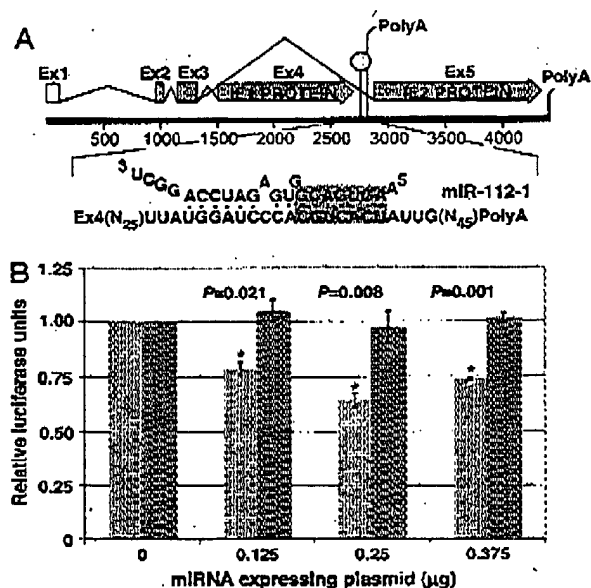
We applied the algorithm to all 3' UTRs of the HCMV genome, and Table 1 shows the 10 most probable miRNA-target pairs of the 4896 total possible miRNA-3' UTR pairs. For each pair, the table shows the score  $PV_{SH}$  and the statistical significance  $PV_{MH}$  of all predictions up to this one. For instance, the 10th miRNA-target prediction, miR-UL112-1 targeting the IE transactivator protein 1 mRNA (IE1, encoded by the UL123 ORF, highlighted), has a score  $PV_{SH} = 10^{-2.21} = 0.0062$  and  $PV_{MH} = 0.125$ , meaning only 12.5% of randomly generated genomes have top ten  $P$  values better or equal to  $PV_{SH} = 10^{-2.21}$ . For top 25 most probable miRNA-target pairs, see SI Table 5C. In fact, the data set in that table suggests that the most significant predictions are the top 10 listed in Table 1 because there is a sharp increase in  $PV_{MH}$  from the 10th to 11th prediction:  $PV_{MH}(10) = 0.125$  and  $PV_{MH}(11) = 0.309$ . Naturally,

$PV_{MH}(k)$  increases toward 1 for larger  $k$ . In our analysis, we required that a target be conserved in six sequenced strains of HCMV. If conservation among strains is not taken into account,  $PV_{MH}$  suggests that there are many more significant targets (35 with  $PV_{MH} < 0.20$ , see SI Table 5C). Finally, the  $PV_{MH}$  values listed in Table 1 are conservative upper bounds because we considered all published sequences of detected potential miRNAs although several are only slight variations of each other and some others are perhaps not real miRNAs.

**HCMV IE1 Protein Synthesis Is Suppressed by miR-UL112-1.** Inhibition of any of the genes in Table 1 could potentially favor latency, but we considered IE1 to be a prime target, given its central role at the start of the HCMV transcriptional cascade. IE1 is one of two main products of the HCMV major IE locus, the other being IE2. IE1 and IE2 are required to execute the virus' transcriptional program (23), and they almost certainly influence the choice between latency and lytic replication. A mutant virus unable to produce a functional IE1 protein replicates efficiently only after infection at a high input multiplicity (24); at lower multiplicities, it fails to accumulate normal levels of early mRNAs (25). It activates transcription at least in part by controlling histone modifications (26).

The algorithm predicted a single binding site for miR-UL112-1 within the 99 nucleotide 3' UTR of the IE1 mRNA. To test the prediction that miR-UL112-1 inhibits translation of IE1 protein, we prepared two reporter constructs. The first contained the wild-type IE1 3' UTR downstream of the luciferase coding region and the second contained a derivative of the 3' UTR lacking the 7-nucleotide seed sequence predicted to be the target of the miRNA (Fig. 1A, shaded sequence). HEK293T cells were cotransfected with set amounts of the reporter plasmids and increasing amounts of an effector plasmid expressing the miR-UL112-1 precursor hairpin sequence. The miRNA induced a statistically significant reduction in luciferase expression from the reporter with a wild-type IE1 3' UTR (maximum repression = ~60%) but not from the modified 3' UTR lacking the seed sequence (Fig. 1B), arguing that miR-UL112-1 targets the seed sequence within the IE1 3' UTR to reduce translation or degrade the RNA.

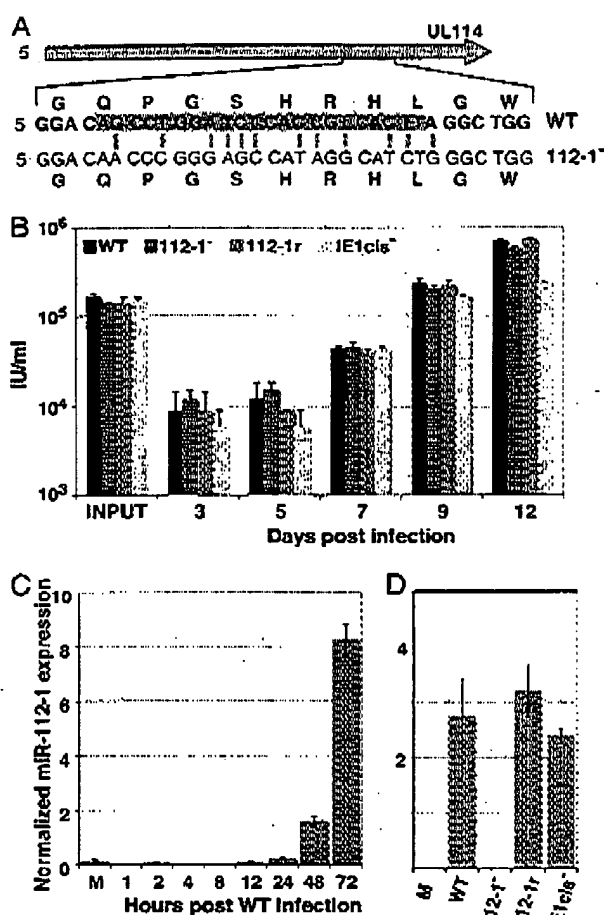
We next generated three viruses to test whether miR-UL112-1 targets IE1 expression within an HCMV-infected cell. The first, BFXΔIE1cis<sup>-</sup>, lacks the 7-nt seed sequence within the IE1 3' UTR that is targeted by the miRNA. The second, BFXsub112-1<sup>-</sup>, is unable to express the miRNA. The miR-UL112-1 precursor is encoded on the DNA strand opposite UL114, and disruption of this ORF inhibits virus replication (27, 28). Consequently, we substi-



**Fig. 1.** miR-UL112-1 inhibits expression from a reporter mRNA containing the IE1 3' UTR. (A) The predicted miR-UL112-1-binding site within the HCMV major IE locus. At the top of the diagram, the spliced mRNAs that encode IE1 and IE2 are depicted with the noncoding exon 1 (Ex1) shown as an open box and the coding exons (Ex2-5) depicted as gray boxes. IE1 and IE2 share Ex2 and Ex3. The PolyA sites and the location of the miR-UL112-1 binding site in the 3' UTR (gray pinhead) are shown. At the bottom of the diagram, the IE1 3' UTR sequence is expanded and the putative miRNA/mRNA base pairing is depicted. The gray box denotes nucleotides within the miRNA seed sequence. (B) Reporter assay for miR-UL112-1 function. 293T cells were cotransfected with firefly luciferase expression plasmids containing either the wild-type (light gray) or mutant IE1 3' UTR (dark gray) as well as a *Renilla* luciferase internal control. Cells were additionally cotransfected with the indicated amounts of a miR-UL112-1 expressing plasmid, and transfection mixtures were balanced with the expression plasmid lacking an insert. Firefly luciferase units were normalized to *Renilla* luciferase. The luciferase units are shown relative to the amount of luciferase from the reporter construct in the absence of miRNA expression plasmids. Asterisks denote *P* values <0.05 as determined by the Student's *t* test.

tuted 12 nucleotides within the miR-UL112-1 precursor sequence while maintaining the coding sequence of the UL114 ORF (Fig. 2A). The miR-UL112-1 mutation was repaired in the final virus, BFXsub112-1r, to control for potential off-target mutations. The viruses grew normally in fibroblasts (Fig. 2B). We also monitored accumulation of miR-UL112-1 by quantitative RT-PCR. The miRNA accumulated to a detectable level between 8–12 h after infection with wild-type virus and then increased as the infection progressed (Fig. 2C). No miR-UL112-1 was detected at 48 h after infection with BFXsub112-1<sup>-</sup>, a time at which the miRNA was readily detected in cells infected with the other viruses (Fig. 2D).

To determine whether IE1 protein levels were affected by the expression of miR-UL112-1, we prepared extracts from infected cells after a 1-h <sup>35</sup>S-labeling period at 6, 24 and 48 hpi with wild-type or mutant viruses. We did not monitor cells later than 48 hpi, even though the miRNA accumulated to higher levels at 72 hpi (Fig. 2A), because infected cells show severe cytopathic effect at the later time. We first examined the steady state levels of several proteins by Western blot assay (Fig. 3A Top). Tubulin levels, which are not altered by infection, provided a precise measure of the amount of cellular protein analyzed in each sample; and the accumulation of the late HCMV protein, pp28, confirmed that all infections progressed normally. We monitored IE1 steady state levels, but little

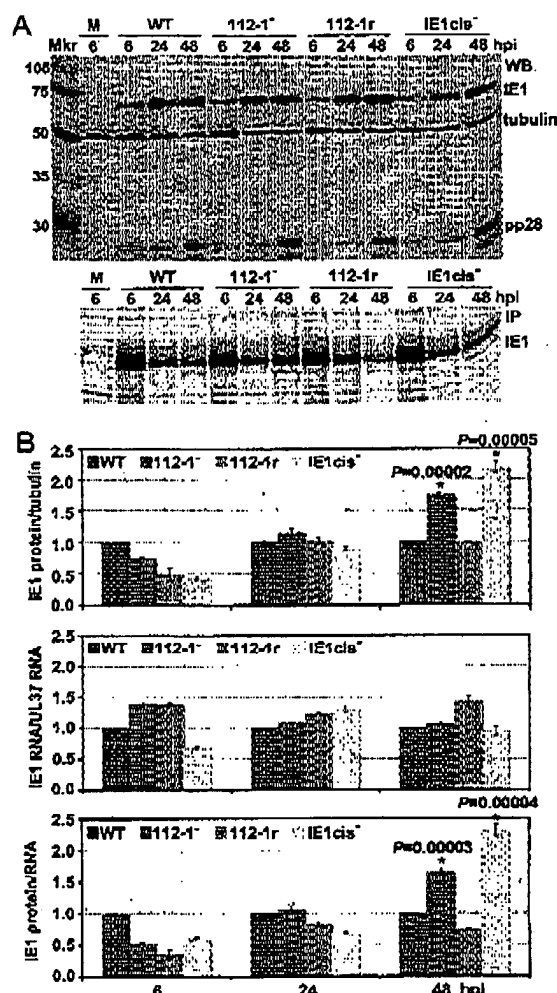


**Fig. 2.** BFXsubUL112-1<sup>-</sup> and BFXdIE1cis<sup>-</sup> grow with wild-type kinetics. (A) The nucleotides mutated in the BFXsubUL112-1<sup>-</sup> (112-1<sup>-</sup>) relative to BFXwt (WT) are shown with gray lines and the complement of the mature miRNA is shaded within the WT sequence. The substituted nucleotides in 112-1<sup>-</sup> conserve the amino acid sequence (shown above and below nucleotide sequences) of the UL114 gene. (B) MRC-5 fibroblasts were infected at a multiplicity of 0.1 pfu/cell with WT, 112-1<sup>-</sup>, BFXsub112-1r (112-1r, a revertant of 112-1<sup>-</sup>) or BFXdIE1cis<sup>-</sup> (IE1cis<sup>-</sup>, lacks the 7-nucleotide seed sequence in IE1 mRNA). Virus (IU, infectious units) was assayed at the indicated times. (C) qRT-PCR with a miR-UL112-1 specific probe was used to detect the accumulation of the miRNA in WT-infected MRC5 fibroblasts over time. miR-UL112-1 levels were normalized to the expression of a cellular small nuclear RNA, hsn68. M, mock infected. (D) Accumulation of miR-UL112-1 was assayed by qRT-PCR at 48 hpi with the indicated viruses and normalized to hsn68 expression. The 112-1<sup>-</sup> virus had miRNA levels similar to mock infected cells.

difference was evident after infection with wild-type and mutant viruses. This was not surprising, because IE1 protein has a >20-h half life, and it accumulates to a high level before the miRNA is available.

Next, IE1 was immunoprecipitated from extracts and subjected to electrophoresis to identify protein synthesized during each 1-h labeling period (Fig. 3A Bottom). The rate of IE1 synthesis was substantially greater at 6 hpi than at later times for all viruses, and this is expected because the promoter responsible for the production of IE1 mRNA is repressed late after infection (29, 30). Radioactivity in the IE1-specific band was quantified relative to the level of tubulin, and Fig. 3B Top presents the results of two





**Fig. 3. Viruses that lack miR-UL112-1 or its binding site synthesize more IE1 protein.** (A) MRC5 fibroblasts were mock-infected (M) or infected with BFXwt (WT), BFXsub112-1<sup>-</sup> (112-1<sup>-</sup>), BFXsub112-1<sup>r</sup> (112-1<sup>r</sup>), or BFXd/IE1cls<sup>-</sup> (IE1cls<sup>-</sup>). Cells were <sup>35</sup>S-labeled for 1 h before harvesting at the indicated times after infection. Lysates were prepared and analyzed by Western blot for IE1, the late virus-coded pp28 or tubulin (Upper) or immunoprecipitation followed by electrophoresis for <sup>35</sup>S-labeled IE1 (Lower). The experiment shown is a representative of 6 independent immunoprecipitations. (B Top) Quantification of <sup>35</sup>S-labeled IE1 relative to tubulin. IE1 protein levels were quantified by phosphorimager analysis of immunoprecipitated complexes from two independent experiments, each of which was analyzed by three independent immunoprecipitations, such as that displayed in A lower. The levels of IE1 protein were normalized to tubulin levels from the Western blot in A. The mutant and revertant viruses are normalized to WT levels for each time point. *P* values were determined by the Student's *t* test. (B Middle) Quantification of IE1 RNA relative to UL37 RNA by qRT-PCR. Mutant and revertant viruses are normalized to WT levels for each time point. (Lower) ratio IE1 protein (from Top) to IE1 RNA (from Middle).

independent experiments, each analyzed by performing three independent immunoprecipitations. At 6 and 24 hpi, we did not observe an effect attributable to miR-UL112-1 activity, consistent with the observation that the miRNA is not detected at 6 hpi and relatively little is present at 24 hpi. In contrast, at 48 hpi, when the miRNA has accumulated to higher levels, the miR-UL112-1-deficient and the IE1 target site-deficient mutants exhibited statis-

tically significant increases (~2-fold) in IE1 protein synthesis relative to the wild-type and revertant viruses.

At each time protein extracts were prepared, total RNA was isolated from a duplicate sample, and the amount of IE1 RNA was determined relative to the level of an independent IE RNA (UL37) by quantitative RT-PCR. IE1 RNA levels varied little among the viruses (Fig. 3B Middle), arguing that the miRNA does not significantly alter the stability of IE1 mRNA and supporting the conclusion that the changes in IE1 protein levels result from the inhibition of translation. The ratio of IE1 protein to RNA was calculated (Fig. 3B Lower), confirming a significant increase in protein synthesis when either the miRNA or its target site is disrupted.

**Predictions of Targets for miRNAs Coded by Other Herpesviruses.** Encouraged by the experimental verification of our prediction in HCMV, we applied the algorithm to an analysis of three additional human herpesviruses. HSV-1, EBV, and KSHV each proved to encode miRNAs predicted to inhibit the expression of viral proteins, including IE proteins. Table 2 displays the rank of the IE-targeting miRNAs among all possible miRNA-3' UTR pairs (the total number is equal to the number of 3' UTRs times the number of miRNAs). The rank is again based on the *P* value *PV*<sub>SH</sub> computed according to the local first order MM or the global third order MM. ICP0 in HSV-1, BZLF1 and BRLF1 in EBV, and Zta and Rta in KSHV are among the virus-specific targets most likely to be targeted by virus-coded miRNAs (top 0.5–2% of virus-specific targets). The BZLF1/BRLF1 3' UTR of EBV is predicted to be targeted by two miRNAs.

SI Tables 3–6 show three additional pieces of information for each virus: First, there is a list (SI Tables 3A–6A) for each miRNA of the total actual and predicted number of binding sites across all 3' UTRs with associated *P* value. miRNAs with smaller *P* value are more likely to regulate some (unspecified) viral genes. The total number of functional binding sites for miRNAs can be estimated from the difference of the total numbers of actual and predicted seed binding sites (21). It is reassuring that this number is always positive, regardless of the virus or the MM used. Second, there is a list (SI Tables 3B–6B) of the top 25 3' UTR targets, sorted according to the *P* value based on the total actual and predicted binding-site counts across all miRNAs. 3' UTRs with small *P* value are likely to be regulated by some combination of viral miRNAs. Third, there is a list (SI Tables 3C–6C) of the top 25 miRNA-3' UTR pairs. Pairs with small *P* value are most likely to be functional pairs. The ranks of the IE genes in Table 2 are derived from this list.

Besides the IE genes, the top predicted miRNA targets include many genes involved in viral DNA replication as well as several inhibitors of apoptosis and other genes involved in immune evasion. Brief descriptions of the predicted targets in these functional groups are summarized in SI Table 7.

## Discussion

Our computational analysis predicts that miRNAs encoded by members of three herpesvirus subfamilies target viral IE genes. HSV-1 ( $\alpha$ -herpesvirus) miR-LAT likely targets ICP0. HCMV ( $\beta$ -herpesvirus) miR-UL112-1 targets IE1. EBV ( $\gamma$ -herpesvirus) miR-BHRF1-3 and miR-BART15 likely target BZLF1 and BRLF1, which share their 3' UTR (Table 1 and SI Table 4C). If the effect of all EBV miRNAs is combined, this 3' UTR is in fact the top target in the whole genome (SI Table 4B). In addition, the seed binding is actually longer than the heptamer 2–8 used in the analysis; it is an octamer 1–8 for miR-BHRF1-3, and if noncanonical binding U-G is allowed, a 9-mer 210 for miR-BART15. KSHV ( $\gamma$ -herpesvirus) Rta (ORF 50) and Zta (ORF K8) are homologues of EBV BRLF1 and BZLF1, respectively, although functionally BZLF1 seems to correspond more to the Rta. The 3' UTR shared by Zta and Rta is likely targeted by the KSHV miR-K10-6-3p, which is numerically the strongest prediction of this algorithm (99.93

Table 2. Whole genome ranks for predicted miRNA-IE target pairs in four herpesviruses

Virus	3' UTR*	Length	miRNA	Seed	Count	Rank A†	Percentile	Rank B
HSV-1	ICP0	186	hsv1-miR-LAT	3-8	1	4 of 154	97.40	12 of 154
EBV	BZLF1, BRLF1	53	ebv-miR-BART15	2-8	1	3 of 2,720	99.89	4 of 2,720
EBV	BZLF1, BRLF1	53	ebv-miR-BHRF1-3	2-8	1	4 of 2,720	99.85	3 of 2,720
HCMV	IE1	92	hcmv-miR-UL112-1	2-8	1	10 of 4,896	99.80	9 of 4,896
KSHV	Zta, Rta	1,144	kshv-miR-K12-6-3p	3-8	4	1 of 1,394	99.93	1 of 1,394

The table reports the top miRNA-IE target pairs for HSV-1, EBV, KSHV, and HCMV after sorting by  $PV_{SH}$  score.

\*BZLF1 and BRLF1 as well as Zta and Rta give rise to 3' coterminal transcripts and therefore genes in each pair have the same 3' UTRs.

†Rank A (resp. rank B) denotes the rank among all possible miRNA-3' UTR pairs sorted by  $P$  values computed for the random sequence based on the first-order (resp. the third-order global) MM. Percentile corresponds to Rank A.

percentile). Finally, besides the IE genes, the analysis (SI Tables 3–6) suggests numerous other likely miRNA targets, e.g., the HSV-1 neurovirulence factor  $\gamma$ 34.5, whose RNA contains an octamer seed binding site for miR-LAT.

We confirmed the predicted inhibition of HCMV IE1 translation by miR-UL112-1 within transfected cells by using reporter constructs (Fig. 1) and within virus-infected fibroblasts by analyzing mutant viruses (Figs. 2 and 3). As we were writing this manuscript, Gray *et al.* (31) reported the same conclusion. They demonstrated that addition of miR-UL112-1 RNA to cells before infection inhibited IE1 expression and viral DNA replication.

Given the broad range of predicted targets (SI Tables 3–6), we anticipate that herpesvirus-coded miRNAs exert regulatory effects directly on viral gene expression during replication and spread within infected hosts. This regulation could have many consequences, e.g., down-regulating viral genes as the infectious cycle progresses to avoid toxicity and helping to modulate viral gene expression to optimize replication in a variety of different cell types. Most importantly, however, our results also suggest that virus-coded miRNAs could play a central role in the establishment and maintenance of latency. Because they target IE products that act at the top of the lytic cascade, miRNAs expressed in cells destined for a latent infection can potentially antagonize the cascade and thereby favor entry into latency. Furthermore, miRNAs expressed during latency could help to prevent reactivation by inhibiting translation of IE transactivators.

There are several indirect justifications of this model. First, although expression of HCMV-coded miRNAs during latency has not yet been evaluated, HSV-1, EBV, and KSHV miRNAs are transcribed and available to function during latency. Second, if miRNAs are indeed central agents responsible for the maintenance of latency, this would explain the mysterious failure to detect proteins in the latent stage of HSV-1. Finally, our algorithm predicts that numerous early genes involved in DNA replication are targeted by viral miRNAs. In fact, the EBV miR-BART2 cleaves the mRNA of the DNA polymerase BALF5 (32). Early genes are normally activated by IE products, and their activity in turn would be expected to importantly influence the choice between lytic replication and latency.

In sum, antagonism of IE genes, which form a control node at the top of the herpesvirus lytic cascade, could contribute importantly to the establishment of latency and seems an optimal strategy for maintenance of latency.

## Materials and Methods

**Computation.** To determine the likelihood that a particular miRNA-3' UTR pair is functional, we computed the corresponding probability  $PV_{SH}$ . Let  $c$  denote the actual count of seed  $n$ -mers in the 3' UTR of length  $l$  and  $p$  the probability (based on the MM described in *SI Materials and Methods*) that any given  $n$ -mer in the random background sequence is the seed  $n$ -mer. Then our  $P$  value  $PV_{SH}$  gives the probability of finding at least  $c$  seed  $n$ -mers in a background sequence of length  $l$  which is equal to the  $P$  value of the binomial distribution,

$$PV_{SH} = PV_{bin}(l - n + 1, c, p)$$

$$= \sum_{i=c}^{l-n+1} \binom{l-n+1}{i} p^i (1-p)^{(l-n+1)-i}$$

In practice,  $l$  is of the order of 100 or 1,000. For a hexamer seed sequence ( $n = 6$ ), a typical  $p$  is  $1/4^6 = 1/4,096$  (exactly if all hexamers were equally likely) and therefore a typical  $c$  is zero, making the equation above impractical. An alternative exact expression for  $PV_{SH}$ , which is numerically efficient is

$$PV_{SH} = PV_{bin}(l - n + 1, c, p) = \frac{B(p, c, l - n - c + 2)}{B(c, l - n - c + 2)},$$

where  $B(x, a, b)$  is the incomplete beta function and  $B(a, b)$  is the usual beta function,

$$B(x, a, b) = \int_0^x u^{a-1} (1-u)^{b-1} du,$$

$$B(a, b) = B(1, a, b).$$

The statistical significance of the top miRNA-target pairs was evaluated by calculating probability  $PV_{MH}$ . Because the majority of  $P$  value  $PV_{SH}$  is equal to 1, we cannot use the standard method of estimating the false discovery rate (22). Instead, we use the following Monte Carlo procedure: First generate  $n - 1,000$  random genomes analogous to the actual genome of interest. This means that each genome will have exactly the same number of 3' UTRs as the genome of interest and each generated 3' UTR will be of the same length as the corresponding real 3' UTR. Each random 3' UTR is generated by using the  $k$ th order MM based on the composition of the corresponding 3' UTR in the real genome.

For each of the  $N$  randomly generated genomes, we repeat the same analysis of computing  $PV_{SH}$  as we did for the real genome; i.e., we compute the score  $PV_{SH}$  for each miRNA-3' UTR and sort them. Now, evaluate the statistical significance of the top  $t$  miRNA-target pairs for the actual genome by counting the number  $N_t$  of the randomly generated genomes in which the  $t$ th top microRNA-3' UTR pair has  $PV_{SH}$  smaller than the  $t$ th pair in the actual genome. For each  $t$ , compute the  $P$  value  $PV_{MH}(t)$  corrected for multiple hypothesis testing by

$$PV_{MH}(t) = \frac{N_t}{N}.$$

$PV_{MH}(t)$  is the probability of finding better scores for the top  $t$  potential microRNA-3' UTR pairs in a random genome with similar properties as the actual genome. The smaller  $PV_{MH}(t)$ , the higher the chance that the predicted targets are real targets.

Additional details relevant to computation are described in *SI Materials and Methods*, including sequences used in the analysis, choice of the seed sequence, construction of random background sequence, use of conservation between strains of species.

**Cells, Viruses, and Plasmids.** MRCS and HEK293T cells were propagated in medium with 10% FBS or 10% newborn calf serum, respectively.

The wild-type virus used in these studies is BFXwt-GFP. It is a derivative of a bacterial artificial chromosome (BAC) clone of the HCMV VR1814 clinical

isolate (33) in which a green fluorescent protein (GFP) expression cassette has been inserted upstream of the US7 ORF. Three derivatives of BFXwt-GFP were produced by using *galK* selection and counter selection to modify BAC DNAs (34) as described in *SI Materials and Methods*. BFXdIE1ds<sup>-</sup> lacks the 7-nt seed sequence for miR-112-1 within the IE1 3' UTR. BFXsub112-1<sup>-</sup> contains 12 single base pair substitutions that block expression of miR-112-1. BFXsub112-1r is a repaired derivative of BFXsub112-1<sup>-</sup>. Virus was generated by electroporation of MRCS cells with BAC DNA (20 µg) plus an HCMV pp71-expressing plasmid (pCGNpp71). Virions were purified by centrifugation through a 20% sorbitol cushion. Virus titers were calculated by infecting fibroblasts and counting IE2-positive foci at 24 hpi.

The construction of plasmids is described in *SI Materials and Methods*.

**mRNA and miRNA Quantification.** Real-time RT-PCR was performed on total RNA isolated from the cells by using the mirVana miRNA isolation kit (Ambion), which isolates total RNA while preserving the miRNA population. DNA was removed by using the DNA-free reagent kit (Ambion). Equal aliquots of total RNA were reverse transcribed by using the TaqMan Reverse Transcription kit with random hexamers according to the manufacturer's protocol (Applied Biosystems). To measure mRNA levels, real-time PCR was performed with SYBR green PCR master mix (Applied Biosystems) and primers specific to exon 4 of IE1 (5'-GCCCTCCCTAAGACCAAT-3' and 5'-ATTTCTGGGCATAAGCCATAATC-3') or exon 1 of UL37 (5'-GACGAAGTCCGATGAGGAGGATG-3' and 5'-TGGGACACTGGCGTGTGTTG-3').

To measure levels of miR-UL112-1, a modified TaqMan-based stem loop RT-PCR was performed (35). TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) was used according to the manufacturer's protocol with the following stem-loop oligonucleotide: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCTGATACGACAGCTG-3'. A 1:15 dilution of the product from the reverse transcriptase reaction was used in a TaqMan quantitative PCR along with 1.5 mM of forward primer (5'-GCCCAAGTACGCTGAGAT-3'), 0.7 mM of reverse primer (5'-GTGCAAGGTCCGAGGT-3'), 0.2 mM of TaqMan probe (5'-6FAM-TACGACAGCTGAGT-mgBnFQ) and 1× Universal TaqMan PCR Master mix (Applied Biosystems). The results were normalized by quantifying the levels of

human U68 small nuclear RNA using the RNU68 Taqman control assay (Applied Biosystems).

**Protein Quantification.** MRCS cells were infected at a multiplicity of 3 pfu per cell. Cells were starved for methionine and cystine before labeling by incubating for 1 h in medium with 10% dialyzed FBS. EasyTag Express Protein Labeling mix (100 µCi; PerkinElmer) was added to the cells for 1 h, after which the labeling medium was replaced with medium containing 10% FCS for 10 min to allow stalled translation to complete. Cells were washed in PBS and then lysed in buffer containing 20 mM Tris acetate pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, and 1% Triton X-100. One tablet of Complete Mini Protease inhibitor (Roche Applied Science) was added per 10 ml of lysis buffer. Protein concentration was calculated by Bradford assay.

Aliquots (10 µg) were subjected to Western blot assay using monoclonal antibodies specific for HCMV IE1 (1812), HCMV UL99 (1084) and monoclonal anti-tubulin antibody (Sigma-Aldrich). An anti-mouse HRP conjugated antibody was used along with the ECL plus detection kit (Amersham) to detect specific bands. Chemiluminescence was analyzed by using a PhosphorImager and ImageQuant TL software (GE Healthcare Life Sciences, Piscataway, NJ).

For immunoprecipitation assays, aliquots of lysate (5 or 10 µg protein) were preadsorbed with Protein A/G Plus Agarose beads (Santa Cruz Biotechnology) for 4 h at 4°C. Anti-IE1 monoclonal antibody (1812) and Protein A/G Plus Agarose were added to the supernatant, which was incubated overnight at 4°C with shaking. Immunoprecipitated complexes were washed three times with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with Complete Mini Protease inhibitor. Beads were boiled in 2× SDS loading buffer and run on an 8% SDS/PAGE gel to separate the immunoprecipitated complexes. Gels were dried and exposed to a phosphor screen, which was analyzed by using a PhosphorImager and ImageQuant TL software.

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